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Article (Accepted Version)

Grima, Dominic P, Sullivan, Melanie, Zabolotskaya, Maria V, Browne, Cathy, Seago, Julian, Wan, Kay Chong, Okada, Yoshio and Newbury, Sarah F (2008) The 5'-3' exoribonuclease pacman is required for epithelial sheet sealing in Drosophila and genetically interacts with the phosphatase puckered. Biology of the Cell, 100 (12). pp. 687-701. ISSN 0248-4900

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The 5' – 3' exoribonuclease *pacman* is required for epithelial sheet sealing in *Drosophila* and genetically interacts with the phosphatase *puckered*.

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Running title: Role of *pacman* in thorax closure in *Drosophila*.

Key words: RNA stability, XRN1, exoribonuclease, *Drosophila* development, RNA degradation.

Abstract

Background information.

Ribonucleases have been well studied in yeast and bacteria, but their biological significance to developmental processes in multi-cellular organisms is not well understood. However, there is increasing evidence that specific, timed transcript degradation is critical for regulation of many cellular processes, including translational repression, nonsense-mediated decay and RNA interference. The *Drosophila* gene *pacman* is highly homologous to the major yeast exoribonuclease *XRN1* and is the only known cytoplasmic 5' – 3' exoribonuclease in eukaryotes. To determine the effects of this exoribonuclease in development we have constructed a number of mutations in *pacman* by P-element excision and characterised the resulting phenotypes.

Results.

Mutations in *pacman* result in flies with a number of specific phenotypes such as low viability, dull wings, crooked legs, failure of correct dorsal/thorax closure and defects in wound healing. The epithelial sheet movement involved in dorsal/thorax closure is a conserved morphogenetic process which is similar to that of hind-brain closure in vertebrates and wound healing in humans. Since the JNK signaling pathway is known to be involved in dorsal/thorax closure and wound healing, we tested whether *pacman* affects JNK signaling. Our experiments demonstrate that *pacman* genetically interacts with *puckered*, a phosphatase that negatively regulates the JNK signaling pathway.

Conclusions

These results reveal that the 5' – 3' exoribonuclease *pacman* is required for a critical aspect of epithelial sheet sealing in *Drosophila*. Since these mutations result in specific phenotypes our data suggest that the exoribonuclease Pacman targets a specific subset of mRNAs involved in this process. One of these targets could be a member of the JNK signaling pathway, although it is possible that a parallel pathway may instead be affected. The exoribonuclease *pacman* is highly conserved in all eukaryotes, therefore it is likely that it is involved in similar morphological processes such as wound healing in human cells.

Introduction

The control of mRNA stability is now widely recognised as a key component in the regulation of gene expression. Since the abundance of any given RNA depends upon the rate that it is transcribed, versus the rate of specific degradation, control of RNA stability is a key factor in the control of gene regulation (Caponigro and Parker, 1996; Cooperstock and Lipshitz, 1997; McCarthy, 1998; Wilusz et al., 2001). In multi-cellular organisms, it is increasingly evident that differential regulation of mRNA stability is crucial for normal embryonic development. For example, in all metazoans studied to date, mRNAs provided maternally which are critical in determining axis formation during early embryogenesis are specifically degraded at a particular point in development (Bashirullah et al., 2001; Cooperstock and Lipshitz, 1997; Tadros et al., 2003). In addition, crucial protein gradients in early *Drosophila* embryos are often dependent on translational suppression of particular target RNAs, such as *nanos* and *hunchback*, followed by their degradation (Macdonald and Smibert, 1996). Therefore transcript degradation during development can be selective and also modulated, suggesting a not well studied control of gene expression during development.

In the yeast *S. cerevisiae*, the major degradation pathway is in a 5' – 3' direction. The critical exoribonuclease in this pathway is Xrn1p, which degrades mRNAs after they have been decapped (Johnson, 1997; Meyer et al., 2004; Muhlrad et al., 1994; Parker and Song, 2004). Mutations in *XRN1* lead to a number of phenotypes including larger cell size, increased doubling times, defective sporulation and sensitivity to the microtubule-depolymerising drug benomyl (Decker and Parker, 1993; Decker and Parker, 1994; Johnson, 1997; Muhlrad et al., 1994; Sweet et al., 2007). Deletion of *XRN1* in yeast is deleterious, but not lethal, because mRNAs can still be degraded by the 3'-5' pathway, via the exosome (Jacobs Anderson and Parker, 1998). Xrn1 is not only involved in the normal decay of mRNA but is required in the major pathway in nonsense-mediated decay (Gatfield et al., 2003), RNA interference (Orban and Izaurralde, 2005) and degradation via microRNAs (Souret et al., 2004; Valencia-Sanchez et al., 2006). Mutations in XRN1 in yeast or silencing of *xrn-1* in *C. elegans* leads to phenotypic defects suggesting that this enzyme is required for normal cellular processes (Decker and Parker, 1993; Decker and Parker, 1994; Johnson, 1997; Johnson and Gray, 1991; Muhlrad et al., 1994; Newbury and Woollard, 2004). Previous work has shown that Xrn1p forms a multicomponent complex with the decapping proteins Dcp1p and Dcp2p, the Lsm proteins, the mRNA degradation factor Pat1p and the DEAD box-containing helicase protein Dhh1p (Bouveret et al., 2000; Parker and Song, 2004; Tharun and Parker, 1998). The majority of these proteins are highly conserved in all eukaryotes studied and are located in cytoplasmic particles known as P-bodies (processing bodies) (Eulalio et al., 2007; Parker and Sheth, 2007; Sheth and Parker, 2003). P-bodies have also been observed in human and mouse cells and these also function as specific sites of mRNA storage as well decay (Bashkirov et al., 1997; Cougot et al., 2004).

The *Drosophila* gene *pacman* is a functional homologue of *S. cerevisiae* Xrn1p. *Drosophila pacman* can complement the exonucleolytic activity of yeast Xrn1p and purified *pacman* protein can degrade nucleic acids in a 5' – 3' direction (Chernukhin et al., 2001; Till et al., 1998). The *pacman* gene encodes a 184kD protein (5.2 kb cDNA) and includes a perfect trinucleotide repeat $(CAG)_n$ encoding polyglutamine. Expansions of polyglutamine repeats have been implicated in a number of human genetic diseases including Huntingdon's disease (MacDonald et al., 2003). The *pacman* transcripts are differentially expressed during development being strongly expressed during oogenesis and during early embryonic development (0-8 hours) with particularly abundant expression in the germ band. The *pacman*

gene is located on the X chromosome at 18C7 (Flybase., 1996; Till et al., 1998).

In this paper, we have analysed the effect of *pacman* on *Drosophila* development. We constructed a number of mutations in the *pacman* gene and analysed the resulting phenotypes. Remarkably, these mutations resulted in specific defects in dorsal and thorax closure as well as wing and leg phenotypes. Pacman also affects wound healing, a morphological process that is similar to thorax closure. We also demonstrate that *pacman* interacts genetically with *puckered*, a phosphatase in the JNK pathway which is known to be critical in thorax closure, dorsal closure and wound healing. Our results show that *pacman* has a specific role in developmental processes in *Drosophila* and suggest that it has, directly or indirectly, a regulatory role on the conserved JNK pathway or acts in a parallel pathway.

Results

Construction of *pacman* mutations

In order to understand the role of *pacman* in *Drosophila* development, we used a reverse genetics approach. All available strains carrying a P-element transposon located near *pacman* (*pcm*) were first checked by inverse PCR. The nearest P-element *P{EP}EP1526* was located 584nt downstream of the *pacman* polyA site, between *pacman* and the convergent gene Nat1 (CG12202) (Flybase., 1996). Since there were no existing mutations within *pacman* at that time, we used P-element excision of P element EP1526 in stock 11456 as the starting stock to construct a number of mutant alleles. After mobilisation of the P-element, 345 excision lines were checked by PCR for deletion events. A total of 7 homozygous viable lines were identified with a deletion in *pacman*. Sequencing of these lines revealed 3 unique excisions which were predicted to encode truncated *pacman* proteins and where the downstream gene was untouched (Figure 1, panel A). Nine lethal lines proved to include larger deletions, which, by Southern blotting experiments and by further PCR experiments showed no evidence of lethality due to *pacman*, therefore were not further studied.

In order to analyse the expression of *pacman* protein in wild-type and mutants, we raised an antibody to *pacman* protein. This polyclonal antibody is highly specific to Pacman in that it recognises a major band of 184kD and is the only band detected which increases in intensity when *pacman* is over-expressed in transgenic flies (data not shown). The minor bands seen in wild-type males and females are most likely due to protein modifications or specific cleavages – we have no evidence for alternative splice forms. The expression of Pacman from flies carrying mutant alleles was tested by Western blotting using these polyclonal antibodies. Homozygous pcm^3 and pcm^6 adults produced truncated protein at levels significantly lower than wild-type whereas pcm^5 animals expressed undetectable levels of *pacman* protein. (Figure 1, panel B). We therefore decided to concentrate on the strongest allele, pcm^5, for further analysis. In all these mutants, there was no major change in expression of the convergent downstream gene Nat1 (CG12202), as detected by semi-quantitative RT-PCR (Figure 1, panel C).

The exoribonuclease *pacman* affects viability

To determine the zygotic effect of *pacman* on viability we crossed *pcm* heterozygous females (pcm^5/FM7c) with pcm^5 hemizygous males (pcm^5/Y) and counted the numbers of the resulting female offspring. If *pacman* has no effect on viability, we would expect equal numbers of homozygous female offspring (pcm^5/pcm^5) and heterozygous female offspring ($pcm^5/FM7c$). At 19ºC and 25ºC this cross resulted in similar numbers of homozygous and heterozygous females (Figure 2A), showing that, at these temperatures, *pacman* mutations have little effect

on adult viability, presumably due to residual activity of the Pcm truncated protein. However, reducing the dosage of the *pcm*^{5} mutant allele from 2 to 1 in hemizygous females carrying the deficiency Df(1)JA27 results in significantly reduced zygotic viability. The deficiency Df(1)JA27 removes 400kb of genomic sequence including the *pacman* gene as well as 50 neighbouring genes on the X chromosome (Flybase). When *pcm*^{5} hemizygous males (*pcm*^{5}/Y) were crossed with heterozygous Df(1)JA27/FM7c females only 10% of the female offspring were of the *pcm*^{5}/Df(1)JA27 genotype compared to 90% *pcm*^{5}/FM7c female siblings at 19ºC (50% of each are expected) (Figure 2B). At 25ºC, this *pacman* allele has little effect on zygotic viability showing that it, along with our other alleles, *pcm*^{3} and *pcm*^{6} are cold temperature sensitive (data not shown). This effect is not due to haplo-insufficiency of the Df(1)JA27 chromosome as offspring from the cross Df(1)JA27/FM7c x +/+ result in approximately equal numbers of Df(1)JA27/+ and FM7c/+ female offspring (ratio of 1.05: 1 observed; 1:1 expected (n = 117). These results therefore suggest that the *pcm*^{5} mutation has a deleterious zygotic effect on development at low temperatures. They also show that the *pcm*^{5} allele has some residual activity and is not a null allele.

Expression of *pacman* cDNA rescues the mutant phenotypes.

To confirm that the observed effects on viability were due to mutations in the *pacman* gene rather than another mutation on the X-chromosome, we generated transgenic flies carrying a full length cDNA copy of *pacman* in order to rescue the mutation. The 5.2 kb *pacman* open reading frame, plus 5' and 3' untranslated regions were cloned into a vector carrying a P-element transposon, and expressed under the control of the heat-shock promoter hsp70Bb pP{CaSpeRhs-*pcm*}). Transgenic flies were then generated using standard techniques. To confirm the expression of the *pacman* cDNA, transgenic flies and embryos carrying this *pacman* construct were subjected to heat-shock (37ºC for 30 minutes, followed by 1 hour recovery at 20ºC) and the expression analysed by Northern and Western blotting. These experiments confirmed that *pacman* was expressed at high levels at the RNA and protein levels both in adults and embryos following heat shock (data not shown). However, these experiments also showed that expression of this *pacman* transgene was leaky in that it was ubiquitously expressed at low (near wild-type) levels throughout the life-cycle at 19ºC. To determine whether these low levels of ectopic expression of *pcm* could rescue its associated mutant defects, the viability of females hemizygous for *pcm*^{5} (*pcm*^{5}/Df(1)JA27;; +/+) was compared to these mutants carrying the *pcm* transgene (*pcm*^{5}/Df(1)JA27;; hs-*pcm*/+). As shown in Figure 2C, ectopic expression of *pacman* completely rescued the viability defects associated with the *pacman* mutation at 19ºC. These data therefore show that the deleterious effects on development at low temperatures are associated with the *pcm*^{5} allele. These experiments also demonstrate that low levels of ectopic expression of *pacman* do not affect viability in this transgenic (compare *pcm*^{5}/FM7c;; +/+ with *pcm*^{5}/FM7c;; hs-*pcm*/+, (Figure 2, panel C)).

pacman adults have wing abnormalities and defects in thorax closure.

Analysis of *pcm*^{5} mutant adult flies revealed a number of interesting defects. These adults had "dull" wings which were often crumpled along the posterior edge with occasional blisters in the posterior half of the wing (Figure 3, panels B and E). Figure 3, panel A shows a *pcm*^{5} male with "dull" wings on the right compared to a male where the *pacman* mutation was rescued by a wild-type copy of *pacman* (*pcm*^{5}/Y;;hs-*pcm*/+) on the left. A mutant female *pcm*^{5}/Df(1)JA27 with crumpled wings is shown on the right of panel 3B compared to a heterozygous female (*pcm*^{5}/FM7c) on the left. For *pcm*^{5}/ *pcm*^{5} females, the penetrance of the "dull wing" phenotype was 98% at 19ºC (n = 295) and 68% at 25ºC (n=200), again showing that this *pacman* allele is temperature sensitive. The dull wing phenotype was fully penetrant

at all temperatures in hemizygous females (pcm^5/Df(1)JA27). Close inspection of the dull wings in these mutants revealed that the surface of the wing was not smooth, but had a regular pattern of raised tissue under each trichome (Figure 3, panel D). This uneven surface would account for the lack of irridescence in the wings of these mutants. This dull wing phenotype was fully rescued by ectopic expression of the wild-type *pacman* gene (Table 2).

In addition to the above defects, many mutant flies also had "crooked" legs, where the 3rd pair of legs was bent within the femur (Figure 3, panel G). This defect appeared to reflect a weakness in the legs as these crooked legs were not observed in newly eclosed flies. The penetrance of this phenotype varied with temperature and again was more penetrant at lower temperatures (Table 1). These phenotypes were completely rescued by ectopic expression of *pacman* as described above. Blistered wings and crooked legs are common phenotypes in flies carrying mutations in genes encoding adhesion proteins or extracellular matrix proteins such as laminin (Brown et al., 2000; Henchcliffe et al., 1993) or in the focal adhesion protein paxillin (Chen et al., 2005).

The most striking effect of this *pacman* mutation was that of a cleft thorax phenotype (Figure 4, panels B, C and D compared to wild-type, panel A). At 25ºC, females hemizygous for pcm^5 (pcm^5/Df(1)JA27) exhibited a cleft thorax phenotype with a penetrance of 11.6% (Table 1). At lower temperatures, no cleft thorax phenotypes were seen but dissection of pupal cases in the large number of dead pupae revealed such severe cleft thorax defects that they had died before emergence. Homozygous or hemizygous mutants (pcm^5/pcm^5, pcm^5/Y) showed a lower frequency of the cleft thorax defect. The severity of this phenotype varied from mild, where there was a bald stripe along the dorsal midline of the thorax, through medium, where the two halves of the thorax had not con-joined, to strong, where the thorax and scutellum were extremely disorganised or one half of the thorax was completely missing (Figure 4, panel D). These phenotypes were completely rescued by the ectopic expression of *pacman* in the transgenic described above. These cleft thorax phenotypes strongly resemble that of flies carrying mutations in *hemipterous* and *kayak*, which are members of the c-Jun N-terminal Kinase(JNK) signaling pathway. Incomplete penetrance and variable expressivity are also observed in *hep* and *kay* mutants (Agnes et al., 1999; Zeitlinger and Bohmann, 1999; Zeitlinger et al., 1997).

The thorax of *Drosophila* is formed during pupariation when the distal tips of the two imaginal wing discs grow together and fuse along the dorsal midline. The cells at the distal tips of the discs become more elongated as they move together by crawling over the underlying layer of larval cells. The leading cells then seal along the dorsal midline (Martin-Blanco, 2000; Usai and Simpson, 2000; Zeitlinger and Bohmann, 1999). Incorrect fusion leads to the cleft thorax phenotype. If *pacman* is involved in this epithelial spreading we would expect it to be expressed in 3rd instar larvae and early pupae, when this epithelial sheet spreading is taking place. Our Western blotting experiments show that *pacman* protein is indeed expressed in 3rd instar wandering larvae, and early, mid and late pupae with higher expression of Pacman at these stages than in 2nd instar larvae and late pupae (Figure 4, panels E and F). Therefore the expression of *pacman* protein is consistent with a role in this process.

If *pacman* is required for thorax closure it would also be expected to be necessary for correct dorsal closure, which is a similar morphological process. Our results show that it is indeed required for this process. Figure 4, panel G shows that hemizygous pcm^5 mutant embryos (pcm^5/ *Df(1)JA27/FM7c*) at 19ºC have a severe "dorsal open" phenotype, which is very similar to that seen in a $kayak^1$ (DFos) mutant (Zeitlinger et al., 1997) (Fig. 4, panel H). This

phenotype results from the two epithelial sheets completely failing to join, resulting in the epithelial sheet springing back to give a wrinkled scrap of tissue. The penetrance of this phenotype in *pcm*^{5}/ *Df(1)JA27/FM7c* embryos raised at 19ºC is 80%. At 25ºC, these embryos show a severe dorsal closure defect resulting in a large anterior hole (Fig. 4, panel J), very similar to that of a *bsk*^{2} (JNK) mutant (Fig. 4, panel K). The dark field image of these embryos shows that the denticle belt patterning is not unduly perturbed (Fig. 4, panels M and N). The "cleft thorax", "dorsal open" and dorsal closure mutant phenotypes provide powerful evidence that *pacman* plays a role in the cell movement, cell adhesion or cell shape change and show an unexpected link between RNA stability and morphogenesis.

pacman affects wound healing.

The epithelial sheet movement involved in thorax closure is a conserved morphogenetic process which is similar to that of dorsal closure in *Drosophila*, hind-brain closure in vertebrates and wound healing in humans (Jacinto et al., 2001). Since *pacman* is highly conserved in eukaryotes, and epithelial sheet sealing is a conserved morphological process, we reasoned that *pacman* might also affect wound healing. To test this, we wounded adult 3-5 day old females on the underside of the abdomen using a small scalpel, being careful not to damage the underlying tissues. The survival of these flies was then compared to controls. Figure 5 shows that *pcm*^{5} and *pcm*^{3} homozygotes survived much less well than controls. For example, the half-life of *pcm*^{5} homozygotes at 25ºC after wounding was 11.2 hours compared to 22.4 hours in controls (Figure 5, panel B). The survival of unwounded *pcm*^{5} flies was similar to unwounded controls over the time course of the experiment (Figure 5, panel F). The survival rate of the controls may reflect the severity of the wounding procedure or could be as a result of infection (although sterile scalpel blades were used). Statistical analysis of the survival curves confirmed that survival at 48 and 72 hours was significantly different for *pcm*^{3} and *pcm*^{5} homozygotes compared to controls ($p<0.0001$ for all comparisons).

In order to determine whether this difference in survival of *pacman* mutants after wounding might be particular to the epithelial sheet sealing process rather than a general metabolic defect, we also followed the formation of the melanin clot over the wound. The clotting process in *pcm* mutants followed a similar time course to that of controls (Figure 5, panels C and D). Therefore these results show that *pacman* is required for the normal wound healing process.

pacman interacts with *puckered*, a phosphatase in the JNK signaling pathway.

The cleft phenotypes observed closely resemble that observed in flies mutant for genes such as *hemipterous* (JNKK), *basket* (JNK) and *kayak* (D-Fos), which are members of a conserved JNK signaling pathway (Agnes et al., 1999; Zeitlinger and Bohmann, 1999). This JNK signaling pathway has been shown to be crucial in controlling the cellular events that govern epithelial sheet sealing processes such as thorax closure, dorsal closure and wound healing (Jacinto et al., 2002; Martin and Parkhurst, 2004; Martin and Wood, 2002; Wood et al., 2002). Therefore *pacman* could be involved in the regulation of thorax closure and wound healing by modulating the expression of gene(s) in this pathway. A potential candidate gene is *puckered*, a crucial phosphatase in this pathway, which dephosphorylates the central JNK, *basket*. We reasoned that the phenotypes we observed could be due to mis-regulation of *puckered* expression by *pacman*.

In order to test this hypothesis, we looked for a genetic interaction between *puckered* and *pacman*. The *puckered* allele *puc*^{A251} is a result of a P-element insertion into the second intron of the *puckered* gene (Martin-Blanco et al., 1998) and are homozygous lethal. The P-element

inserted (P[w$^+$, lacZ]A251.1) carries a lacZ reporter gene which has been used to monitor the transcriptional activity of the *puc* promoter (Martin-Blanco et al., 1998). Flies carrying both pcm^5 and puc^{A251} alleles were generated and the phenotypes examined. Male and female flies homozygous (or hemizygous) for *pacman* and heterozygous for puc^{A251} (pcm^5/ pcm^5;; puc^{A251}/TM6b,Tb,Sb and pcm^5/Y;; puc^{A251}/TM6b,Tb,Sb) were viable but showed a number of interesting phenotypic defects. In 58% of males and 70% of females raised at 19ºC, there was a "bald" patch at the anterior end of the dorsal midline, indicating that thorax closure was not quite complete (Figure 6, panel B). When these flies were raised at 25ºC, 95% of males and 66% of females had bald patches. All other genotypes had no bald patches (Table 3). Greater than 90% of flies homozygous (or hemizygous) for *pacman* and heterozygous for the *puckered* mutation (pcm^5/ pcm^5;; puc^{A251}/TM6b,Tb,Sb and pcm^5/Y;; puc^{A251}/TM6b,Tb,Sb) had dull wings at 19 ºC and 25ºC indicating that *puckered* did not substantially affect this *pacman* phenotype. This observation therefore suggest that *pacman* affects expression of *puckered*, either by directly regulating the JNK pathway or by regulating a parallel pathway.

Discussion.

In this study we have investigated the effects of a 5' – 3' exoribonuclease *pacman* on *Drosophila* development. This was achieved by constructing mutations in *pacman* and examining the zygotic effect of the resulting phenotypes. Although we did not succeed in generating a null allele, the allelic series we obtained shed light on the function of *pacman* in development. The most striking phenotype observed in *pacman* mutants is a defect in thorax formation such that the two halves of the thorax on the dorsal side do not join together correctly. The dorsal part of the thorax in wild-type *Drosophila* is formed by cells at the tips of the two wing imaginal discs which migrate towards each other over a layer of larval cells and then fuse together along the dorsal midline (Agnes et al., 1999; Martin-Blanco et al., 2000). This epithelial cell movement is very similar to that seen in dorsal closure in *Drosophila*, hind-brain closure in vertebrates, epiboly in Xenopus and in wound healing in humans (Jacinto et al., 2002). We also showed that *pacman* affects wound healing in *Drosophila*, which significantly strengthens our hypothesis that *pacman* has a conserved role in epithelial sheet movement. Furthermore, our results in a previous paper demonstrate that the *pacman* orthologue *xrn-1* in *C. elegans* is essential for ventral enclosure, which has been shown to be a similar morphological process to dorsal and thorax closure in *Drosophila* (Newbury and Woollard, 2004) (Jacinto et al., 2002) although the molecular similarities between the two processes remain controversial. Our results on *pacman* in *Drosophila*, taken together with those on *xrn-1* in *C. elegans*, suggest that the 5' – 3' mRNA degradation pathway is a crucial factor in this morphological process.

Our results presented above, where we have used living organisms, rather than tissue culture cells, suggest that the 5' – 3' mRNA degradation pathway is important for cellular processes. That it is defects in mRNA degradation which lead to the phenotypic defects observed, rather than some other function of Pacman, is suggested by our recent results showing that an exoribonuclease-dead version of Pacman cannot complement the *pacman* mutation (Grima at al. in prep). Our results are in agreement with studies in human tissue culture cells showing that the degradation of unstable RNAs containing AU-rich elements (AREs), such as *c-myc* and *c-fos* is primarily in a 5' – 3' direction (Newbury et al., 2006), emphasising the importance of this pathway in human cells. Earlier studies, using human tissue culture cell extracts, demonstrating that degradation of short-lived mRNAs such as *c-myc* and *c-fos* are primarily in a 3' – 5' direction via the exosome (Mukherjee et al., 2002; Wang and Kiledjian, 2001) can be explained by the fact that cytoplasmic complexes containing the decapping/5' – 3' degradation enzymes are removed or disrupted during preparation of the extract. Therefore

our results support the conclusion that 5' – 3' degradation of mRNAs is important in yeast, *Drosophila* and mammalian cells.

The cleft thorax or dorsal closure phenotypes of *pacman* mutants resemble that of mutations in the JNK signaling pathway. Hypomorphic mutations in *hemipterous*, *basket* and *kayak* or over-expression of the JNK phosphatase *puckered* result in similar cleft thorax defects to those seen in *pacman* mutants. In addition, the JNK pathway, including phosphatase *puckered* is known to be involved in the epithelial sheet sealing pathway during wound healing (Ramet et al., 2002). There are a number of ways in which *pacman* might regulate the JNK pathway. Firstly, Pacman might act directly on *puckered* transcripts, in which case *puckered* transcripts would be expected to be stabilised with a corresponding increase in levels of Puckered protein. This would lead to increased dephosphorylation of its target kinase (the JNK Basket) and downregulation of the JNK pathway resulting in defective epithelial sheet sealing. However, this hypothesis seems unlikely as *puckered* mutations would then be expected to suppress (rather than enhance) *pacman* phenotypes as is seen in hep^{1};; puc^{E69} and kay^{2}/kay^{1};; puc^{E69} double mutants (Zeitlinger and Bohmann, 1999). Alternatively, *pacman* may have its primary effects on other transcripts, which in turn affect the expression of *puckered*. It is also possible that *pacman* may act in a pathway that is parallel, rather than linear to, the JNK pathway.

As well as epithelial sheet sealing, the *pacman* phenotypes such as dull wings and bent legs would suggest that *pacman* also affects transcripts in other cellular pathways. A possible target for *pacman* is *paxillin*, a focal adhesion adaptor. Overexpression of Dpax in flies results in bent and/or twisted leg phenotypes and also blistered wings (Chen et al., 2005). Therefore is possible that the Pacman exoribonuclease directly targets *paxillin* mRNA to ensure that it is expressed at the correct levels in wild-type flies.

Interestingly, we consistently find the phenotypic effects of *pacman* alleles to be more severe in *pacman* mutant females compared to *pacman* mutant males. For example, the enhanced lethality in hemizygous females pcm^{5}/Df(1)JA27 at 19ºC (viability of 2.7% of total offspring) compared to pcm^{5}/Y at 19ºC (viability 13.9% of total offspring) highlights this difference. As yet, we do not understand the differences in phenotypic effects between males and females but suspect it may be to do with dosage compensation. It is possible that *pacman* mutations may affect the levels of non-coding RNAs that are involved in dosage compensation (Deng and Meller, 2006). If this is the case, then *pacman* mutations may also modulate the levels of expression of genes located on the X-chromosome resulting in different phenotypic effects in males compared to females.

How could an exoribonuclease, which is relatively non-specific in-vitro (Chernukhin et al., 2001) selectively affect the expression of particular RNAs such as those encoding proteins involved in thorax formation? Our work, using *Drosophila* neurones (Barbee et al., 2006) shows that Pacman is localised in cytoplasmic particles which appear to be analagous to yeast or human P-bodies. In these particles, Pacman is co-localised with other proteins in the 5' – 3' degradation pathway, such as the decapping enzymes dDcp1 and dDcp2, as well as proteins involved in translational repression (Barbee et al., 2006; Lin et al., 2006). In human tissue culture cells, mRNAs appear to be guided to these P-bodies by RNA binding proteins such as TTP or by micro-RNAs (Kedersha et al., 2005; Lai et al., 2003) We speculate that *pacman* target mRNAs are normally transported to P-bodies and then rapidly down-regulated by translational repression and degradation. Reduction in Pacman activity may interfere with the translational repression process, resulting in inappropriate levels of target proteins and

downregulation of the JNK pathway. The finding that *pacman* regulates the JNK pathway is significant in that it will allow us to identify and define *in-vivo* targets for this 5' – 3' exoribonuclease. This is a crucial step in understanding the mechanisms whereby ribonucleases can specifically target RNAs and is likely to be relevant to other specific degradation events such as those that occur during RNA interference.

Materials and Methods

Drosophila strains, generation of *pacman* mutants and molecular characterisation of mutants.

Fly stocks used were obtained from the Bloomington stock centre unless otherwise indicated. Bloomington stock 11456 carrying the P insertion *P{EP}EP1526* was used to create *pcm* mutants by imprecise P-element excision using standard protocols. Bloomington stock 971 carrying the deficiency *Df(1)JA27/FM7c* was used to generate hemizygous stocks and to generate balanced stocks of the mutagenized *pcm* lines. Potential mutant lines were screened by duplex PCR using primers jls1 5'- TCAAAAAGGCAGTGGCATGAG-3' and jls2 5'-GTCCGAATCTGATGGGGTCT-3' and control primers dob1f 5'-GACATTGTTCAGGGCAAGGCAG-3' and dob1b 5'-GGAGCGGTGAGGTCGTTAAATAC-3'. Mutant lines were then checked by Southern blotting for single transposition events. Mutations were characterised at the molecular level by amplification of DNA at either side of the breakpoint using primers jes1 5'-TCCCGATCACGATGAAGACC-3' and big1 5'-ACTGCCGCCTCAGATCTG – 3' and then sequencing. To test whether the molecular lesions in the nine lethal lines were due to a deletion in the *pacman* gene, DNA isolated from heterozygotes carrying a viable allele of *pacman* (*pcm*^{2} ; encoding a slightly truncated *pacman* protein (truncated by 66 aa) together with each of the lethal alleles was subjected to PCR using the primers jls1 and jls2. Since these primers do not result in any product from the *pcm*^{2} chromosome, any PCR product must come from the chromosome carrying the lethal allele. All these lethal alleles resulted in a PCR product of a size comparable to that from a wild-type chromosome. Therefore the lethality of these 9 alleles did not result from deletions in the *pacman* gene. The predicted sizes and molecular weights of the three truncated proteins encoded by the alleles *pcm*^{3}, *pcm*^{5} and *pcm*^{6} compared to the wild-type protein were as follows: WT Pcm = 1612 amino acids (aa), 184.5 kD; Pcm3 = 1461 aa, 167.7 kD; Pcm5 =1293 aa, 149.2kD; Pcm6 = 1396 aa, 160.0 kD. Unfortunately no null alleles were generated.

To determine whether the deletions within *pacman* affected expression of the downstream gene Nat1 we used semi-quantitative RT-PCR in duplex reactions with primers to the "housekeeping" control rp49 as well as Nat 1 primers. The primers used were: Nat1-34f 5' – CACTACGACTACATGCGCGATA-3', Nat1-34r 5' – GAACTTGGCGCAGATCTCCT -3', rp49F1 5' – CAAGGGACAGTATCTGATG – 3', rp49R1 5'- CAGTAAACGCGGTTCT-3'. Primers were designed to amplify a region of the Nat1 gene spanning the intron between exons 3 and 4 (Nat1-34f and Nat1-34r) in order to distinguish between genomic DNA amplicons (324 bp) and cDNA amplicons (244 bp). RNA was extracted from 15 flies from each of the lines tested using the Qiagen RNeasy extraction kit and cDNA was prepared using the Superscript III reverse transcriptase kit as per manufacturer's instructions. All amplicons first appeared faintly at 24 cycles and clearly at 26 cycles. PCR was repeated using the same program except that only 26 cycles were used. Positive (gDNA from w^{1118}) and negative (template replaced with water) controls were used to validate the results.

The stock over-expressing *pacman* was generated by cloning a full length *Drosophila* cDNA, including the 5'UTR and 3'UTR into the P element transformation vector pP{CaSpeR-hs} followed by germline transformation of w^{1118} flies using standard methods. Three independently generated lines all gave identical results. All whole fly images were captured using the Nikon DN100 camera attached to a Nikon SMZ800 microscope.

Generation of antibodies, Western blotting and immunostaining.

For preparation of an antibody to Pacman, cDNA encoding a 54 kD C-terminal portion of Pacman was expressed as a His-tag fusion protein in *E. coli* using the expression vector pET28a. The histidine tag was removed by thrombin treatment and the *pacman* protein fragment cut from the gel for use in raising antibodies. The rabbit polyclonal antibody was prepared by the company Eurogentec. Although this antibody was made to the C-terminus of the *pacman* protein we know it can detect the truncated *pacman* protein encoded by the pcm^5 mutants for two reasons. Firstly, sequencing of the pcm^5 lesion shows that the truncated protein still includes 140 amino-acids of the C-terminal region used to raise the antibody. Secondly, our immunoprecipitation experiments using testes from pcm^5 mutants have shown that this antibody can immunoprecipitate the truncated *pacman* protein (Zabolotskaya, et al., Biochemical Journal (under revision)).

SDS-PAGE and Western blotting was performed essentially as described (Sambrook et al., 1989). Equal numbers of adult flies, raised at 25ºC, were used in each lane. The binding of the polyclonal antibody to Pcm was detected using the Amersham ECL Western blot reagent kit. Primary polyclonal anti-Pcm antibody was used at 1:2000 and the monoclonal anti-actin antibody (Sigma) at 1:10,000. The secondary antibodies were monoclonal anti-rabbit HRP conjugated antibody (Sigma) and monoclonal anti-mouse HRP conjugated antibody (Sigma) both used at 1:80.000. The Western was quantified using ImageJ software.

Cuticle preps

BL971 females (Df(1)JA27/FM7c) were collected as virgins and mated to pcm^5 males in collection cage for 2 days with fresh grape juice agar and yeast supplied every day. Embryos at the appropriate stage of development were dechorionated in 50% bleach for 3 minutes before washing twice in distilled water and transferring to a scintillation vial containing 3ml methanol and 3ml heptane. The vial was shaken vigorously for 1 minute to devitellinise the embryos which then sank to the bottom. The upper layer and interface were siphoned off and the embryos washed twice in methanol. Embryos were then transferred to a clean microscope slide and the methanol allowed to air dry. 50-100µl of Hoyer's medium/Lactate (3:1) was added and a coverslip placed over top of the embryos. The slide was then incubated overnight at 65 °C to clear and then flattened with a brass weight. Slides were then examined and photographed under dark field or bright field microscopy on a Zeiss Axioplan microscope. Similar methods were used for the wild-type and bsk^2 embryos.

Wounding experiments

Female flies were wounded on the ventral side of the abdomen, using a small scalpel, taking care not to damage the underlying tissues and then placed in food vials. Typically, ten flies for each stock were wounded and compared to ten unwounded controls. The number of flies still alive was recorded every 3 hrs (during the day) until at least 50% of the mutant flies were dead. This data was then analysed using the statistics computer programme Minitab. The Chi^2 test was applied to the data to determine whether there was a significant difference between survival of wounded and unwounded flies after wounding. To determine the time of clot formation, 3-4 day-old flies were wounded as above and the progression of clot formation recorded at 10, 20, 25, 30, 35, 40, 50 and 60 minutes.

Acknowledgements

We would like to thank Dr. France Docquier for advice on the P-element excision work and Professor Roy Parker for advice and encouragement. We are also grateful to Dawn Field, Helen Glenwright, Alex Blundell and Sarah Allan for technical help. This work was

supported by the Leverhulme Trust, the UK Biotechnology and Biological Sciences Research Council, the Nuffield Foundation, the Biochemical Society and the Genetics Society.

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Tables

Table 1.

	pcm^5/JA27	
	19^{o}C	25^{o}C
Crooked legs	53.3	9.4
Blistered wings	13.3	1.0
Cleft thorax	0	11.58
Dull wings	100	100
Number scored	243	295

Table showing the percentage of phenotypes of female flies carrying one copy of the mutant *pcm*^{5} allele. These flies resulted from the cross Df(1)JA27/FM7c x *pcm*^{5}/Y. Note that Df(1)JA27/FM7c is not associated with any of these mutant phenotypes.

Table 2.

Parents	*w, pcm*^{5}/FM7c; +/+ x *w*, *pcm*^{5}/Y; *hs-pcm* /+			
Males	*w, pcm*^{5}/Y; +/+		*w, pcm*^{5}/Y; *hs-pcm* /+	
	white eye, dull wing	white eye, shiny wing	orange eye, dull wing	orange eye, shiny wing
	50	0	0	55
Females	*w, pcm*^{5}/FM7c; +/+		*w, pcm*^{5}/FM7c; *hs-pcm* /+	
	white eye, dull wing	white eye, shiny wing	orange eye, dull wing	orange eye, shiny wing
	60	0	0	65

Table showing that ectopically expressed Pacman rescues the “dull wing” phenotype in both male and female flies at 19^{o}C. The offspring were derived from the cross *w, pcm*^{5}/FM7c; +/+ x *w*, *pcm*^{5}/Y; pP{CaSpeRhs-*pcm*} /+. If the ectopically expressed *pacman* (hs-*pcm*) did not rescue the dull wing phenotype we would expect that flies carrying the hs-*pcm* construct (*w, pcm*^{5}/Y; hs-*pcm* /+ or *w, pcm*^{5}/FM7c; hs-*pcm* /+) to have orange eyes and dull wings. Note that this pP{CaSpeRhs-*pcm*} construct is leaky and expresses at low levels even when the flies are not subjected to heat-shock.

Table 3.

		Percent of bald patches for each genotype	
	Genotype	19°C	25°C
1	*pcm*^{5}/*pcm*^{5};;*puc*^{A251}/TM6b,Tb,Sb	70	67
2	*pcm*^{5}/*Y*;;*puc*^{A251}/TM6b ,Tb,Sb	58	95
3	*pcm*^{5}/*FM7i*;;*puc*^{A251}/TM6b ,Tb,Sb	0	0
4	FM7i/Y;;*puc*^{A251}/TM6b ,Tb,Sb	0	0
5	*pcm*^{5}/Y	0	0
6	FM7i/Y	0	0
7	*pcm*^{5}/ *pcm*^{5}	0	0
8	*pcm*^{5}/FM7i	0	0
9	*puc*^{A251}/TM6 ,Tb,Sb	0	0

Table showing the percent of dorsal bald patches in particular genotypes. Flies of the genotypes in rows 1-4 are relevant offspring from the cross *pcm*^{5}/FM7i;;*puc*^{A251}/TM6b,Tb,Sb x *pcm*^{5}/Y;; *puc*^{A251}/TM6b,Tb,Sb. At least 50 of each genotype were scored.

Figure Legends.

Figure 1.
(A) Diagram showing the genomic structure of *pacman*. Exons (■), introns (━), the "domino" repeat (QQEAQ), the polyglutamine repeat (polyQ) and the poly-adenylation site is shown. The insertion site of the P-element used to generate deletions in the *pacman* gene is given, as is the downstream gene CG12202 (Nat1). The *pacman* alleles described in this paper are given below: gaps represent deletions at the genomic DNA level. Arrows indicate sites of the primers used to characterise these mutants. **(B)** Western blotting of *pacman* homozygous and hemizygous mutant adults showing that the truncated proteins are not detectable (pcm^5) or are poorly expressed (pcm^3 and pcm^6). The molecular weights of these truncated proteins are given in the methods section. An actin loading control is shown below. **(C)** Agarose gel showing no significant difference in the mRNA levels of the downstream gene Nat1 between any of the strains tested as determined by semi-quantitative RT-PCR. The first two strains are controls; (w^{1118}) represents the progenitor stock 11456 carries P-element *P{EP}EP1526* inserted between *pcm* and *Nat1*. pcm^5 and pcm^3 are two *pacman* mutants, gDNA represents the genomic DNA control and –ve is the "no DNA" control. Duplex PCR reactions were carried out using Nat1 primers and control rp49 primers and were amplified for 26 cycles.

Figure 2.
Pacman affects zygotic viability. **(A)** Percentages of female offspring of the two different genotypes from the cross pcm^5/FM7c x pcm^5/Y at 19ºC and 25ºC. (n = 460 (19ºC) and 839 (25ºC)**(B)** Percentages of female offspring of the two different genotypes from the cross Df(1)JA27/FM7c x pcm^5/Y showing that, at 19ºC, a single copy of the mutant gene significantly reduces viability compared to female siblings. (n= 143 (19ºC) and 254 (25ºC). (**C**) Ectopic expression of *pacman* rescues *pacman* viability phenotypes. At 19ºC the viability of female flies carrying the *pacman* transgene (pcm^5/Df(1)JA27;; hs-*pcm*/+) is completely rescued compared to *pacman* mutant female siblings (pcm^5/Df(1)JA27;; +/+) (total scored = 458). Percentages relate to percentages of female offspring; 25% of each genotype are expected if there are no effects of *pacman* on viability.

Figure 3.
Wing and leg phenotypes of *pacman* mutant adults. **(A)**: "Dull wing" phenotype of pcm^5 male (right) compared to a mutant male ectopically expressing *pacman* (pcm^5/Y; pP{CaSpeRhs-*pcm*}/+) (left). **(B)**: Dull and crumpled wing phenotype (arrow) of pcm^5 hemizygous female (pcm^5/Df(1)JA27)(right) compared to heterozygous control (pcm^5/FM7c)(left). **(C and D)**: Close-up of the surface of a wild-type wing **(C)** and a pcm^5 "dull" wing **(D)** showing a regular pattern of raised tissue rather than a smooth surface. **(E)**; Example of a blistered wing from a pcm^5/Df(1)JA27 female (arrow). **(F and G)**; Example of a kinked leg (arrow) from a pcm^5 male where the femur of the 3rd leg is bent **(G)** compared to the wild-type control **(F)**.

Figure 4.
Phenotypes of *pacman mutant* flies. **(A)**; Dorsal view of a wild-type thorax. **(B)**; Mutant homozygous pcm^5 and **(C-D);** hemizygous pcm^5 (pcm^5/Df(1)JA27) females showing the cleft-thorax phenotypes. **(E)**; Western blot showing expression of *pacman* protein in wild-type larvae, pupae and adults compared to the Actin loading control. Pacman is expressed at higher levels in L3, early pupae and mid pupae during growth and differentiation of the imaginal discs. L1= 1st instar larvae, L2 = 2nd instar larvae, L3 = 3rd instar larvae, EP = early pupae, MP

= mid pupae, LP = late pupae, M = adult males, F = adult females. **(F)** Quantification of the Western using the actin loading control. **G-O**. Cuticle preparations of mutant and wild-type embryos. (G) Cuticle prep of a *pacman* mutant embryo expressing one copy of the pcm^5 allele (i.e. a hemizygous mutant $pcm^5/Df(1)JA27$ (reared at 19ºC)) compared to a kay^1/ kay^1 homozygous mutant (Zeitlinger et al., 1997) (**H**) and wild-type control (**I**). Note that this *pacman* mutant has a "dorsal open" phenotype resulting from complete failure of dorsal closure and is very similar to that of a homozygous mutant in *kayak*(DFos), encoding a JNK signaling protein. (**J) and (M)** Cuticle prep of a *pacman* mutant embryo expressing one copy of the pcm^5 allele (i.e. a hemizygous mutant $pcm^5/Df(1)JA27$ (reared at 25ºC)) compared to a bsk^2/ bsk^2 homozygous mutant **(K)** and **(N)**. **(J)** and **(K)** show the embryos using bright field microscopy, which emphasises the large dorsal holes (arrows). **(M)** and **(N)** show the same embryos using dark field microscopy, demonstrating that the pattern of denticle belts is not substantially perturbed in these mutants. A wild type embryo, taken using bright field **(L)** and dark field microscopy **(O)** is shown for comparison.

Figure 5.
Survival of *pacman* mutants after wounding. **(A and B)** Average half lives of pcm^3, and pcm^5 mutants after wounding together with isogenic controls. Means and standard errors of at least 3 independent experiments are given. Flies raised at 19ºC (A) and 25ºC (B) were used. In all cases, the *pacman* mutants survived significantly less well than controls. **(C and D)** Average times of clot formation in *pacman* mutants compared to controls in flies raised at 19ºC **(C)** and 25ºC **(D)**. Means and standard errors for at least 3 independent experiments are given. **(E and F)** Typical survival curves for pcm^5 mutants and controls showing survival of wounded and unwounded flies raised at 19ºC **(E)** and 25ºC**(F)**. The survival of unwounded mutants was not significantly different to that of controls over the time period of the experiment. The symbol —●— represents control unwounded, —◆— represents control wounded, —▲— represents pcm^5 unwounded, —■— represents pcm^5 wounded.

Figure 6. Phenotypic defects in flies carrying mutations in *pacman* and *puckered*. **(A)**; Bristles on the dorsal side of the thorax in a wild-type fly. **(B)**; Bald patch at the anterior dorsal midline of a pcm^5/ pcm^5;;puc^{A251}/ TM6b ,Tb,Sb female (arrow).

Figure 1

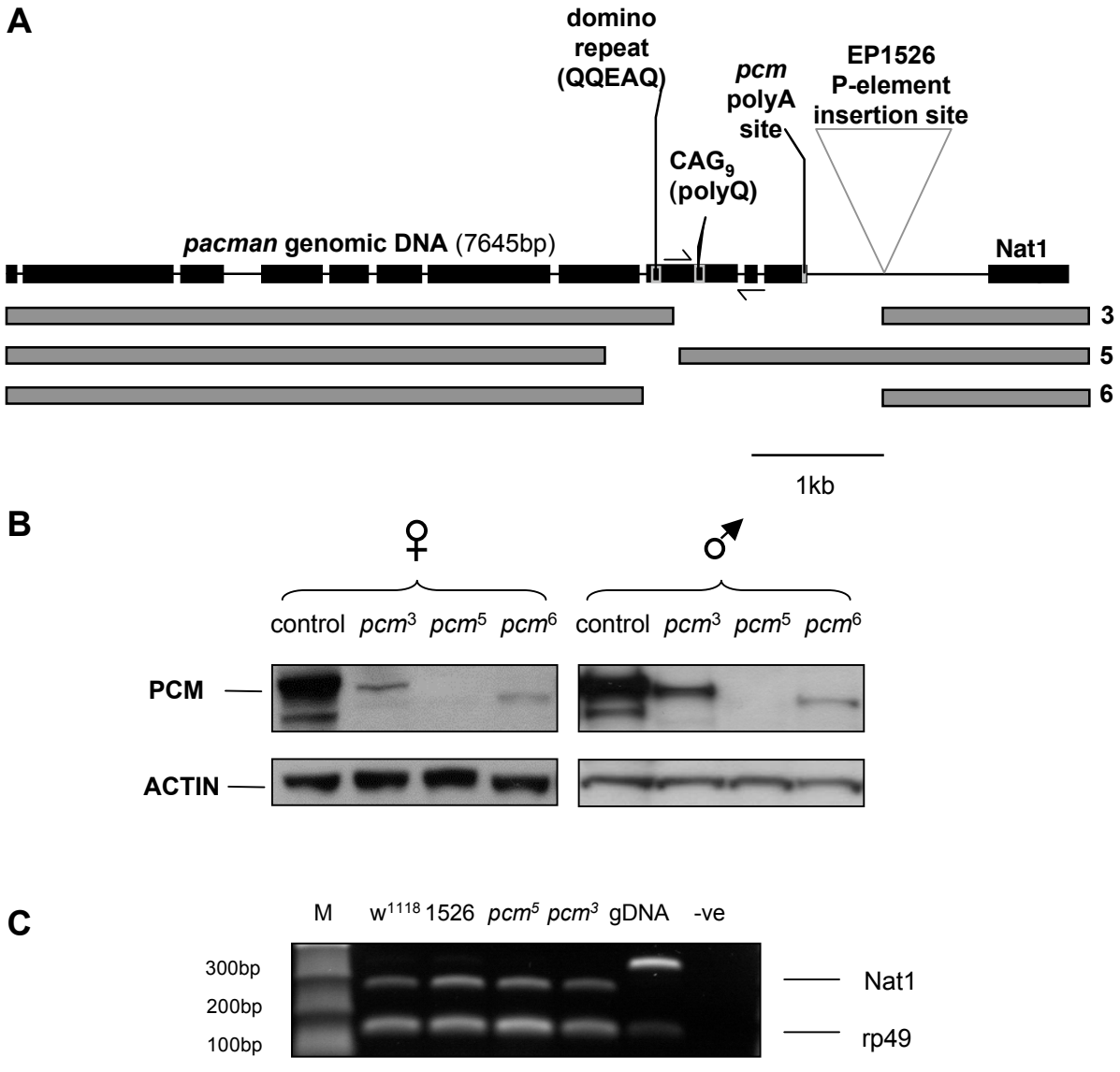

A
domino repeat (QQEAQ)
pcm polyA site
EP1526 P-element insertion site
CAG9 (polyQ)
pacman genomic DNA (7645bp)
Nat1
3
5
6
1kb
B
♀
♂
control pcm3 pcm5 pcm6
control pcm3 pcm5 pcm6
PCM
ACTIN
C
M w1118 1526 pcm5 pcm3 gDNA -ve
300bp
200bp
100bp
Nat1
rp49

Figure 2

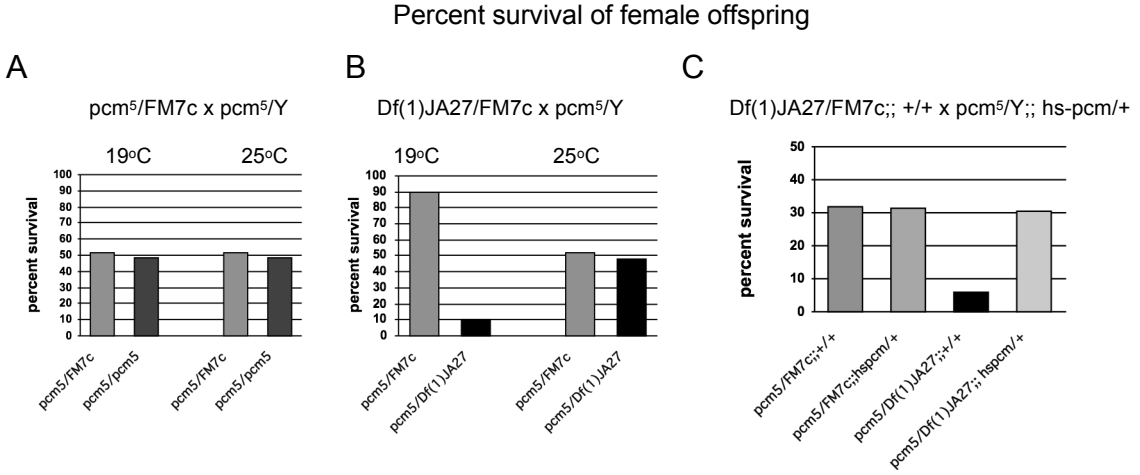

Percent survival of female offspring
A
pcm5/FM7c x pcm5/Y
19oC
25oC
percent survival
pcm5/FM7c
pcm5/pcm5
pcm5/FM7c
pcm5/pcm5
B
Df(1)JA27/FM7c x pcm5/Y
19oC
25oC
percent survival
pcm5/FM7c
pcm5/Df(1)JA27
pcm5/FM7c
pcm5/Df(1)JA27
C
Df(1)JA27/FM7c;; +/+ x pcm5/Y;; hs-pcm/+
percent survival
pcm5/FM7c;;+/+
pcm5/FM7c;;hspcm/+
pcm5/Df(1)JA27;;+/+
pcm5/Df(1)JA27;; hspcm/+

Figure 3

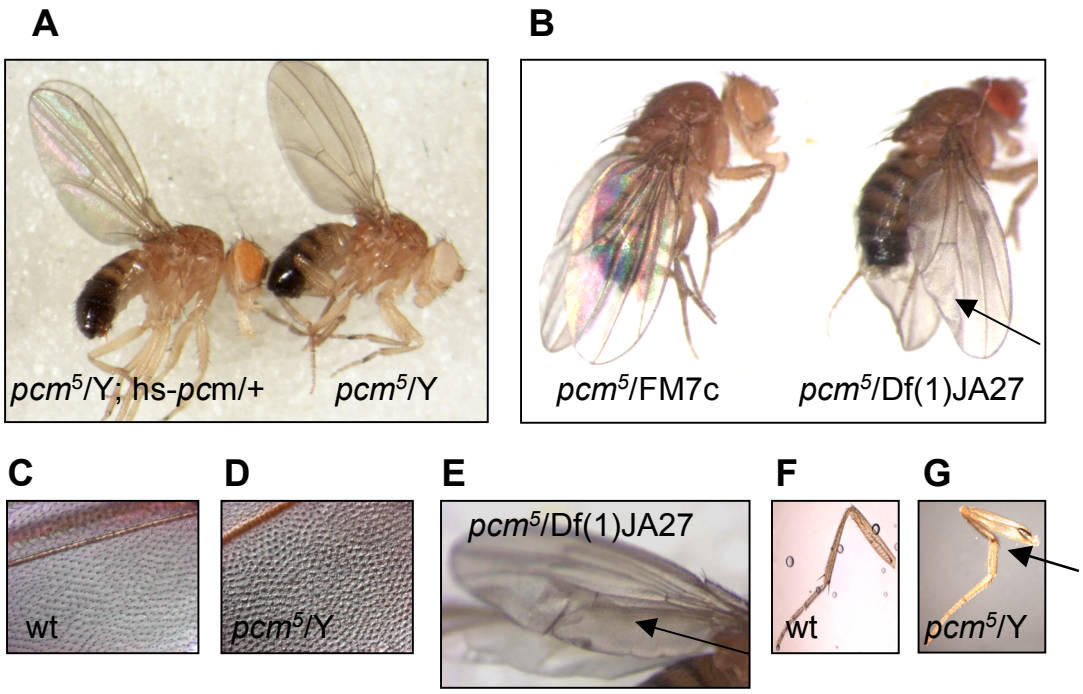

A
B
pcm5/Y; hs-pcm/+
pcm5/Y
pcm5/FM7c
pcm5/Df(1)JA27
C
D
E
F
G
wt
pcm5/Y
pcm5/Df(1)JA27
wt
pcm5/Y

Figure 4

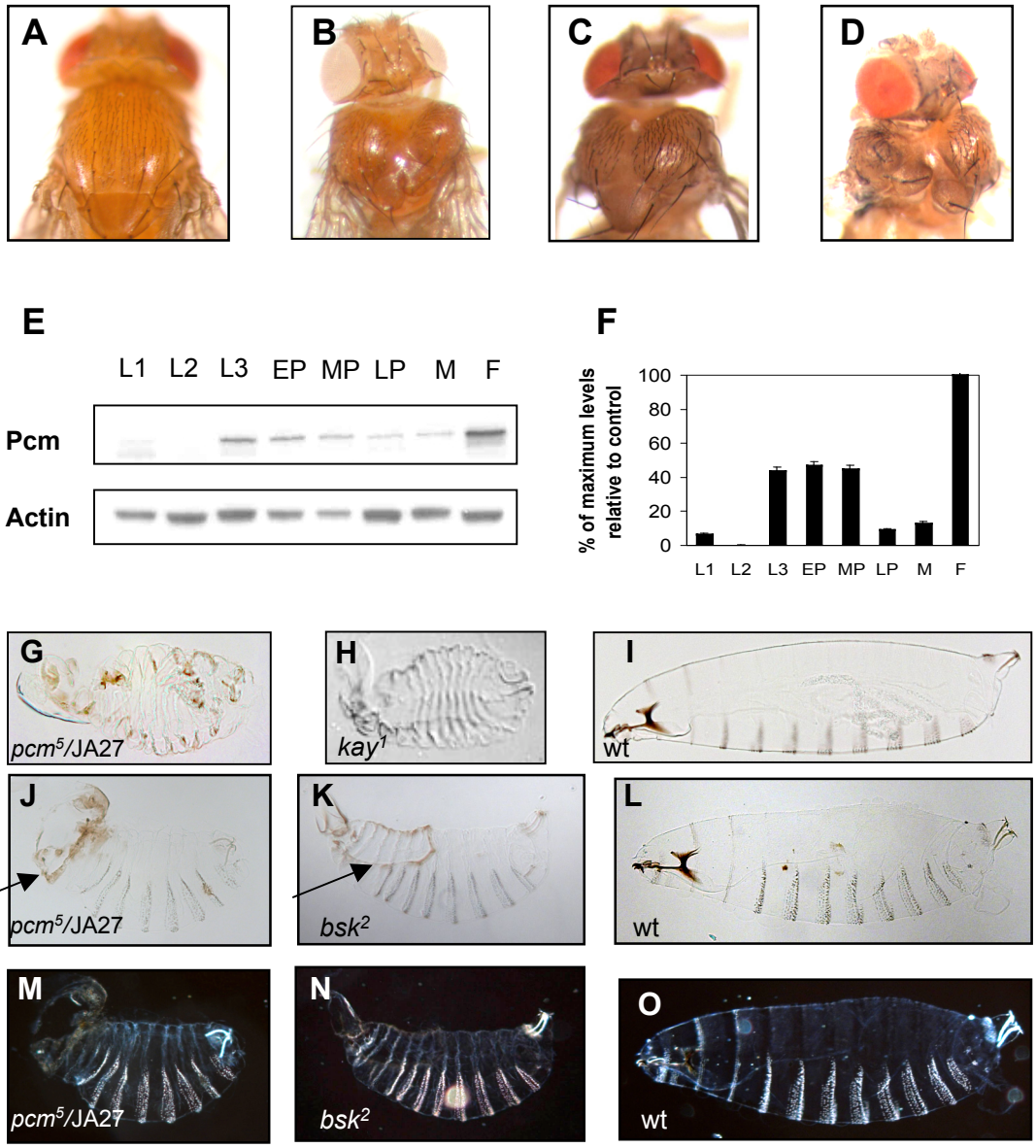
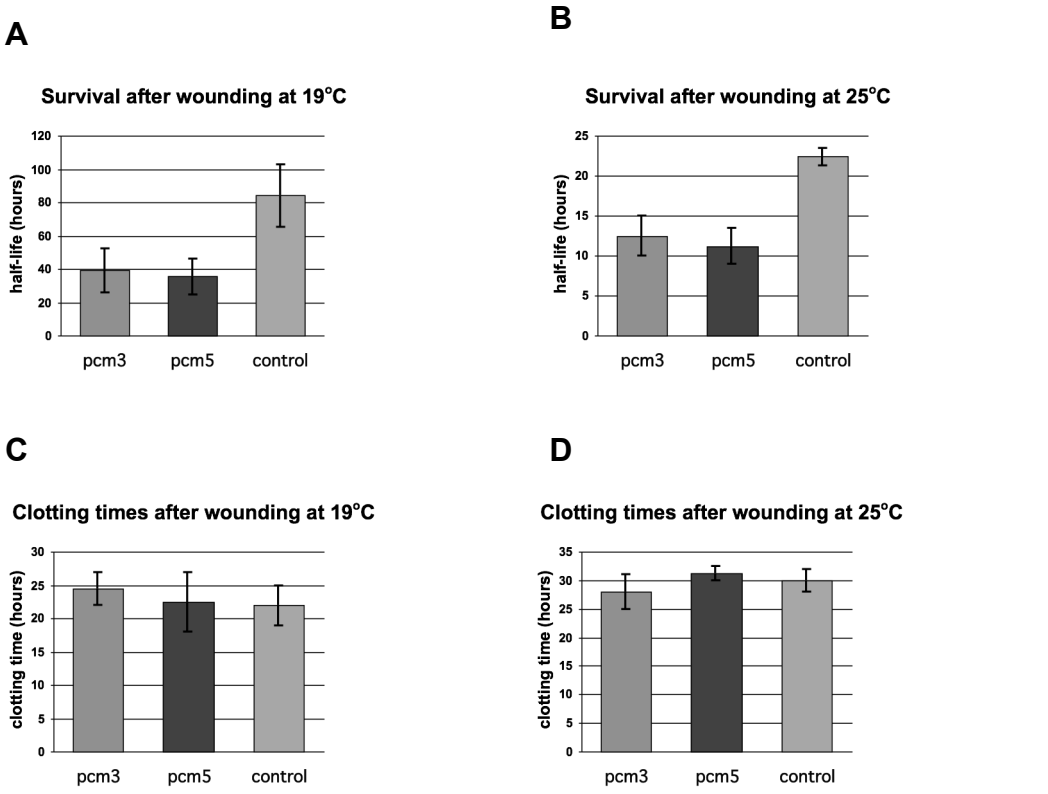
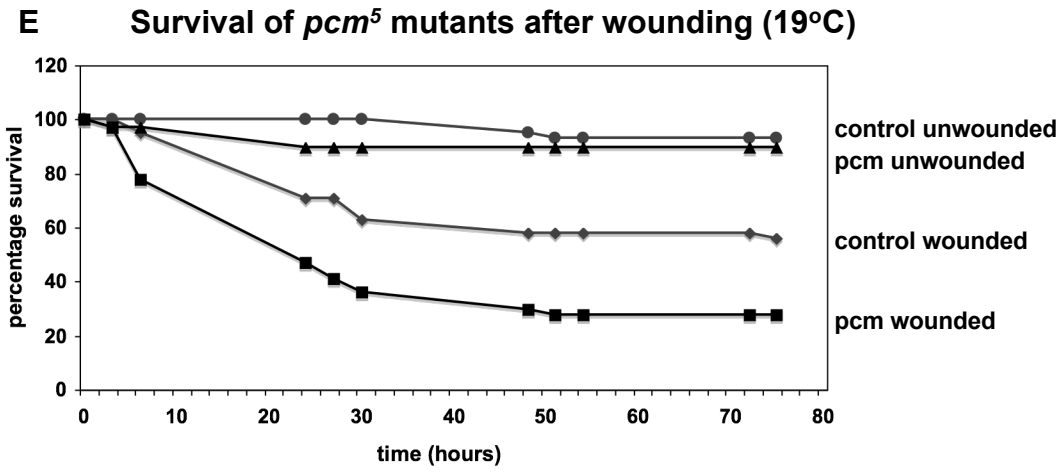

A
B
C
D
E
L1 L2 L3 EP MP LP M F
Pcm
Actin
F
% of maximum levels relative to control
100
80
60
40
20
0
L1 L2 L3 EP MP LP M F
G
pcm5/JA27
H
kay1
I
wt
J
pcm5/JA27
K
bsk2
L
wt
M
pcm5/JA27
N
bsk2
O
wt

Figure 5


A
Survival after wounding at 19°C
half-life (hours)
120
100
80
60
40
20
0
pcm3
pcm5
control
B
Survival after wounding at 25°C
half-life (hours)
25
20
15
10
5
0
pcm3
pcm5
control
C
Clotting times after wounding at 19°C
clotting time (hours)
30
25
20
15
10
5
0
pcm3
pcm5
control
D
Clotting times after wounding at 25°C
clotting time (hours)
35
30
25
20
15
10
5
0
pcm3
pcm5
control


E
Survival of pcm5 mutants after wounding (19°C)
percentage survival
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
time (hours)
control unwounded
pcm unwounded
control wounded
pcm wounded

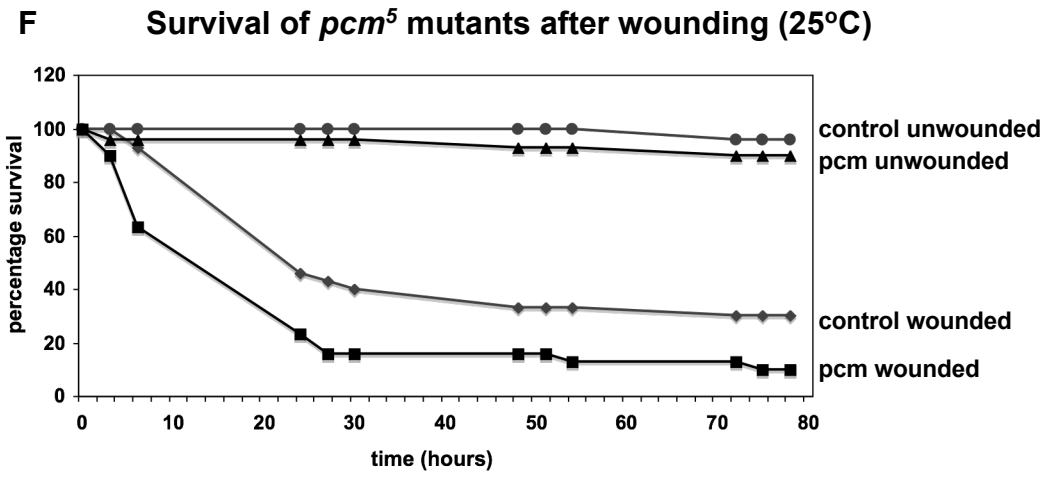

F
Survival of pcm5 mutants after wounding (25°C)
percentage survival
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
time (hours)
control unwounded
pcm unwounded
control wounded
pcm wounded

Figure 6